Influence of N-acetylcysteine against dimethylnitrosamine induced hepatotoxicity in rats

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Abstract
This study evaluates the hepatoprotective and antioxidant properties of N-acetylcysteine (NAC) on dimethylnitrosamine (DMN) induced hepatotoxicity in male Wistar albino rats. A single intraperitoneal dose of DMN (5 mg/kg b.w.) caused a significant increase in the levels of the serum marker enzymes (aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), glutamyl transpeptidase (γ-GT)) and a subsequent decrease in AST, ALT, ALP and increase in LDH and γ-GT in the liver tissue indicating hepatocellular damage. Elevation in the status of lipid peroxidation, fall in the activities of the enzymic (superoxide dismutase, catalase) and non-enzymic antioxidants (vitamin C, vitamin E) in the liver tissue further confirms oxidative stress and hepatocellular damage induced on DMN administration. Oral administration of NAC (50 mg/kg b.w.) for 7 days significantly prevented the above alterations in the status of the marker enzymes of hepatotoxicity and antioxidant parameters and restored them towards normalcy, which was further substantiated by the histopathological studies of the liver tissue. These results suggest that NAC offers hepatoprotection by ameliorating DMN-induced oxidative stress and hepatotoxicity and this protective effect was attributed to its antioxidant and free radical scavenging properties.

Keywords
Dimethylnitrosamine, hepatotoxicity, lipid peroxidation, N-acetylcysteine, oxidative stress.

Introduction
Dimethylnitrosamine (DMN), an N-nitroso alkyl compound, is a known hepatotoxin, carcinogen and mutagen, capable of producing high incidence of tumors in liver, kidney and lungs of rats (Haggerty and Holsapple, 1990). Possible human exposure to DMN occurs through diet, environmental sources and endogenous formation in the stomach. DMN is found in variety of foods such as soybean oil, canned fruits, meat products, bacon, fish, cheese, cured meats, beer and alcoholic beverages. DMN toxicity is of environmental concern because of its presence in tobacco smoke at concentrations ranging from 0 to 140 ng per cigarette (IARC, 1978). It is also found as a contaminant in rubber products at concentrations approximately (1-100 μg/kg; ATSDR, 1989). DMN is metabolized primarily in the liver by the microsomal membrane-bound enzyme cytochrome P₄₅₀ 2E1 to produce highly reactive electrophiles which methylates nucleic acids and proteins leading to carcinogenicity (Yang et al., 1990). DMN has been employed as an experimental tool in the evaluation of hepatoprotective properties of plant extracts such as Sho-saiko-to extract (Kusunose et al., 2002), Lonicera japonica Thunb. (Caprifoliaceae) (Sun et al., 2010) and chemical compounds such as Pirfenidone (Tada et al., 2001) and Malotilate (Ala-kokko et al., 1989) in rats. Hence, the present study was planned to evaluate the...
hepatoprotective efficacy of N-acetylcysteine (NAC) on DMN-induced hepatocellular damage and oxidative stress.

N-acetylcysteine (NAC) is a thiol and a precursor of L-cysteine and reduced glutathione and a direct antioxidant (Zachwieja et al., 2005). NAC first found its use as a mucolytic agent in clinical medicine in 1960s (Ziment, 1978). For number of years NAC has been used as an antidote for overdose of acetaminophen (Flanagan and Meredith, 1991). NAC acts as an antioxidant restoring the pool of intracellular GSH, which is depleted during oxidative stress and inflammation (Sadowska et al., 2007). NAC has been shown to exert its antioxidant effect by scavenging the free radicals such as OH (hydroxyl radical), H$_2$O$_2$ (hydrogen peroxide) and O$_2^-$ (superoxide radical) (Aruoma and Halliwell, 1989; Benrahmoune et al., 2000). As a source of sulfhydryl group it indirectly facilitates reduced glutathione biosynthesis (Ocal et al., 2004). NAC is reported to offer protection against hepatocellular damage induced by various chemical hepatotoxins such as ethanol (Ronis et al., 2005), methanol (Raza et al., 2003) and CCl$_4$ (Galicia et al., 2010).

In this study, we investigated the hepatoprotective and antioxidant effects of NAC against DMN-induced oxidative stress and hepatotoxicity using rats as animal models.

**Materials and methods**

**Animals**

Wistar albino male rats ($200 \pm 20$ g) procured from the institutional animal house facility were used for the study. The animals were randomly divided into four groups with six animals in each and were housed in polypropylene cages over husk bedding. They were provided standard pellet feed and water ad libitum and maintained under controlled environmental conditions (temp: $27 \pm 2^\circ$C; Relative humidity: 50%-70%; 12 h light/dark cycle). All the animal experiments were performed after getting prior approval from the Institutional Animal Ethical Committee (IAEC), governed by strict guidelines prescribed by CPCSEA.

**Chemicals**

Dimethylnitrosamine (DMN) and malondialdehyde (MDA) were purchased from M/s. Sigma-Aldrich Chemicals, Pvt. Ltd., USA. NAC was purchased from M/s. SISCO Research Laboratory, Chennai, India. All the other chemicals used in this study were of analytical grade and were purchased locally.

**Experimental design**

The rats were divided into four groups with six animals in each group. The experimental design is as follows.

Group I: Rats received saline for 7 days and served as control.

Group II: Rats received a single dose of DMN (5 mg/kg b.w; i.p.) on day zero and were left without any treatment for a period of 7 days (Nicolini et al., 1976).

Group III: Rats received a single dose of DMN (5 mg/kg b.w; i.p.) on day zero and NAC (50 mg/kg b.w; p.o) daily for a period of 7 days.

Group IV: Rats received NAC alone (50 mg/kg b.w; p.o) daily for a period of 7 days (Kalaiselvi et al., 2005).

At the end of the experimental period, all the animals were subjected to mild ether anesthesia and blood was collected from retro-orbital plexus and serum was separated by centrifugation at 2500 rpm for 15 min. The animals were killed by cervical decapitation and the liver tissue was excised quickly, washed in ice cold saline and blotted to dryness. A 1% liver tissue homogenate was prepared in Tris-HCl buffer (0.1 M; pH 7.4), centrifuged and clear supernatants separated were preserved at (12 to 15$^\circ$C) in vials for further biochemical analysis. A piece of liver tissue was sectioned out and fixed immediately in phosphate buffered formal saline and stained with hematoxylin-eosin for histopathological analysis.

**Biochemical assays**

**Measurement of marker enzymes of hepatotoxicity.**

The activities of aspartate transaminase (AST) and alanine transaminase (ALT) in serum and liver tissue were estimated according to the method of (Reitman and Frankel, 1957). The activities of alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were estimated according to the methods detailed by (King, 1965a, b), respectively. Gamma glutamyl transpeptidase (γ-GT) was assayed in serum and liver tissue based on the procedure detailed by (Rosalki and Rau, 1972).

**Measurement of enzymic and non-enzymic antioxidants.**

Lipid peroxidation in the liver tissue homogenate was assayed in serum and liver tissue based on the procedure detailed by (Ohkawa et al., 1979).
The activities of enzymic antioxidants superoxide dismutase and catalase were assayed in the liver tissue samples according to the methods of (Marklund and Marklund, 1974) and (Sinha, 1972), respectively. The content of the non-enzymic antioxidants vitamin-C (ascorbic acid) and vitamin-E (α-tocopherol) were determined according to the methods of (Omaye et al., 1979) and (Varley et al., 1976), respectively. Protein was estimated based on the method detailed by (Lowry et al., 1951).

### Statistical analysis

The data was subjected to one-way ANOVA and Tukey’s multiple comparison test was done to evaluate the significance of difference in means between various treatment groups using SPSS statistical package (Version: 7.5). Values are presented as mean ± SD and *p* value < 0.05 was considered significant.

### Results

#### Effect of NAC post-treatment on the levels of marker enzymes of hepatotoxicity in serum and liver tissue of experimental rats

The status of marker enzymes of liver toxicity 7 days after a single dose administration of DMN in serum is presented in Table 1. DMN-induced hepatocellular damage was clearly evidenced by a fourfold increase in the activities of ALT and ALP, threefold increase in AST and LDH and a twofold increase in γ-GT in serum. Post-treatment of NAC significantly prevented this increase in the status of all the marker enzymes in serum and reversed them towards normalcy.

In contrast to this increase, there was a significant decrease in the status of (AST, ALT and ALP; Figure 1), and significant increase in (LDH and γ-GT; Figure 2) in the liver tissue of DMN-administered rats. The above alterations in the status of all these marker enzymes in the liver tissue were effectively prevented by NAC on its post-treatment.

#### Effect of NAC post-treatment on the status of lipid peroxidation in liver tissue of experimental rats

The status of lipid peroxidation in the liver tissue of control and experimental animals are presented in Figure 3. DMN treatment produced a threefold increase in the levels of lipid peroxidation in the liver tissue. NAC post-treatment decreased the elevated lipid peroxidation levels and brought it back towards normalcy.

#### Effect of NAC post-treatment on the status of enzymic antioxidants in liver tissue of experimental rats

Figures 4 and 5 depict the effect of NAC post-treatment on the activities of superoxide dismutase and catalase, respectively, in the liver tissue. DMN

<table>
<thead>
<tr>
<th>Parameters (IU/L)</th>
<th>Group I (control)</th>
<th>Group II (DMN alone)</th>
<th>Group III (DMN + NAC)</th>
<th>Group IV (NAC alone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST</td>
<td>37.94 ± 2.69</td>
<td>110.60 ± 10.25a</td>
<td>55.32 ± 2.82a,b</td>
<td>40.01 ± 2.93</td>
</tr>
<tr>
<td>ALT</td>
<td>40.04 ± 4.79</td>
<td>156.25 ± 10.89a</td>
<td>66.91 ± 3.50a,b</td>
<td>41.30 ± 3.15</td>
</tr>
<tr>
<td>ALP</td>
<td>105.97 ± 10.93</td>
<td>410.07 ± 18.70a</td>
<td>213.32 ± 11.32a,b</td>
<td>110.86 ± 9.22</td>
</tr>
<tr>
<td>LDH</td>
<td>138.12 ± 13.10</td>
<td>412.02 ± 23.03a</td>
<td>223.50 ± 9.55a,b</td>
<td>147.84 ± 9.21</td>
</tr>
<tr>
<td>γ-GT</td>
<td>90.99 ± 6.95</td>
<td>211.30 ± 16.19a</td>
<td>141.02 ± 7.00a,b</td>
<td>89.33 ± 6.54</td>
</tr>
</tbody>
</table>

Note: Results are given as mean ± SD of six rats.

*Significant at *p* < 0.001 when compared to group I.

b Significant at *p* < 0.001 when compared to group II.
administration caused a highly significant 50% reduction in the activities of both superoxide dismutase and catalase in the liver tissue, which was significantly restored back towards normalcy on NAC post-treatment.

**Effect of NAC post-treatment on the levels of non-enzymic antioxidant vitamins in liver tissue of experimental rats**

Figures 6 and 7 show the levels of vitamin C and vitamin E in the liver tissue of control and experimental animals. There was a highly significant 40% to 50% decrease in the levels of these non-enzymic antioxidant vitamins in the liver tissue of DMN-treated rats. Post-treatment with NAC significantly prevented this decrease and restored their levels towards normalcy in the liver tissue. NAC alone administration did not produce any significant change in the status of any of the above parameters studied and were found similar to that of control.
Histopathological examination of the liver tissue

Histopathology of the liver tissue of control and experimental rats are presented in Figure 8. The liver sections of rats treated with DMN alone (group II) shows extensive portal triaditis and abundant infiltration of neutrophils around portal triad area. The chromatin is condensed and more number of hepatocytes shows premalignant changes, indicating hepatocellular damage and onset of carcinogenicity. The sinusoidal space is dilated and is infiltrated by neutrophils. Liver sections of rats treated DMN + NAC (group III) shows extensive sinusoidal dilatation with infiltration of neutrophils. Few hepatocytes show hyperchromatic nucleus and portal triaditis. NAC alone (group IV) treated rats shows normal histological architecture of the liver and was comparable to that of saline-treated control (group I).

Discussion

In this investigation, DMN-induced hepatocellular damage is confirmed by a marked elevation in the levels of serum AST, ALT, ALP, LDH and γ-GT, and a simultaneous fall in the status of AST, ALT and ALP, and increase in LDH and γ-GT in the liver tissue. High concentration of serum transaminases is taken as an index of hepatic injury and it is observed during parenchymal hepatocellular damage induced by drugs and chemicals (Vasudevan and Sreekumari, 2001). Previous studies have shown elevation in the levels of serum AST and ALT on DMN treatment (Wang et al., 2010) and our present observations are in accordance with these reports. The elevated activities of these enzymes are indicative of cellular leakage and loss of the functional integrity of liver cell membranes (Rajesh and Latha, 2004). The elevation in the status of the serum transaminases observed in this study might be due to the release of these enzymes from the cytoplasm into the blood circulation rapidly after the rupture of the plasma membrane and cellular damage caused by the free radicals released during the metabolism of DMN. ALP, a nonspecific membrane-bound enzyme is also considered as a reliable marker of liver damage, which even in a mild elevated state is reported to produce parenchymal liver damage. Increase in the status of ALP, observed in this study, might be suggestive of parenchymal cell damage induced on DMN exposure. LDH is a cytoplasmic enzyme and its activity in serum is found elevated during malignancy by 70% of normal (Tolman and Rej, 1999). γ-GT is the most sensitive indicator of hepatobiliary disease (Vasudevan and Sreekumari, 2001). Gross elevation in the status of γ-GT is an indication of liver metastasis (Szczeklik et al., 1961). The elevated levels of LDH and γ-GT found in this study are an indication of parenchymal cell damage and induction of hepatic necrosis and pre-malignant hepatocellular damage induced by DMN administration. NAC post-treatment significantly prevented these DMN-induced alterations in the status of the marker enzymes in both serum and liver tissue and restored their activities towards normalcy. Thus, from this investigation, it is suggested that, NAC might have effectively attenuated DMN-induced hepatotoxicity by inhibiting liver damage, by maintaining the hepatocellular membrane integrity and by suppressing the leakage of cellular enzymes.

Oxidative stress occurs when the antioxidant defense system is overwhelmed by the excessive production of reactive oxygen species and free radicals (Nencini et al., 2007). Increase in the intracellular levels of reactive oxygen species causes peroxidation of polyunsaturated fatty acids of membrane phospholipids and further reacts with oxygen-forming peroxy radical which in turn reacts with the adjacent side chains and enabling the chain reaction of membrane-bound lipids resulting in lipid peroxidation. Measurement of lipid peroxidation and the altered levels of endogenous scavengers are taken as direct in vivo reliable indices for the contribution of free radical generation and thereby oxidative stress (El-Khatib et al., 2001). In the current study, a three-fold elevation in the status of lipid peroxidation in the DMN exposed rats indicates the formation of reactive oxygen species and oxidative stress. Increased
oxidative stress and lipid peroxidation have been reported in DMN-induced hepatotoxicity (Farombi et al., 2009; George et al., 2004) as well as in diethylnitrosamine (a close analogue of DMN), induced liver injury in rats (Pradeep et al., 2007) and our findings are in accordance with these reports. NAC post-treatment effectively reduced the elevated lipid peroxidation levels in the liver tissue and counteracted the oxidative stress induced by DMN treatment. This shows that NAC might offer protection to the liver against oxidative damage by actively scavenging the free radicals produced by DMN and its toxic metabolites.

Superoxide dismutase and catalase acts mutually to constitute the enzymic antioxidant defense mechanism against reactive oxygen species (Bhattacharjee and Sil, 2006). The activities of these enzymic antioxidants were found decreased by 50% of control levels in the liver tissue of rats administered DMN. This decline in the activities of these enzymic antioxidants could possibly be due to their over utilization towards the suppression of reactive oxygen species and subsequent free radicals that is liberated during metabolism of DMN. In the present study, post-treatment with NAC showed increased activity of these antioxidant enzymes compared to DMN-treated rats, indicating the potentiality of NAC to act as a powerful antioxidant and free radical scavenger by preventing the peroxidative damage caused by DMN. These findings are supported by the investigations conducted by Ozaras et al. (2003) on the ameliorating effect of NAC against alcohol-induced elevation in lipid peroxidation and reduction in superoxide dismutase in the rat liver tissue.

Disruption of oxidant-antioxidant homeostasis and hepatocellular damage was further clearly demonstrated by a fall in the levels of the non-enzymic antioxidant vitamins C and E in the liver tissue of DMN-treated rats. Vitamins E and C are said to act synergistically to scavenge free radicals from the biological system. Vitamin C is a low-molecular weight antioxidant which defends the cellular compartment against water-soluble oxygen and nitrogen radicals (Jurczuk et al., 2007). Vitamin E is a major lipid-

Figure 8. Effect of N-acetylcysteine (NAC) post-treatment on the histopathology of the liver tissue of control and experimental rats (×40).
soluble antioxidant that protects cellular membranes and lipoproteins from peroxidation (Yavuz et al., 2004), and thus minimizes lipid peroxidation in the biological systems (Traber and Atkinson, 2007). Thus, both vitamins C and E have been documented to protect the biological system against oxidative damage induced by xenobiotics (Beutttner, 1993). The decrease in the levels of these non-enzymic antioxidant vitamins observed in the present study could be attributed to its over utilization in scavenging free radicals and thereby preventing oxidative stress and consequent hepatocellular damage induced by DMN treatment. Post-treatment with NAC effectively prevented this DMN-induced decrease in the status of the above non-enzymic antioxidant vitamins and reversed them towards normalcy. NAC exhibits protection against the DMN-induced fall in the levels of these vitamins by virtue of its free radical scavenging and potent antioxidant activity.

The biochemical findings are further supported by the histopathological observations of the liver tissue. The histopathological findings confirmed the establishment of hepatic necrosis and malignant changes upon DMN alone (group II) treatment (Figure 8). George et al. (2004) reported centrilobular congestion, marked dilatation of central vein and sinusoids, massive necrosis and infiltration of neutrophils in rats treated DMN alone, and our present observations are in agreement with these reports. DMN + NAC (group III) treatment showed improvement in the architecture of the liver tissue when compared to DMN alone (group II), indicating the hepatoprotective ability of NAC against DMN induced hepatocellular damage.

Conclusions
In conclusion, our present study shows that NAC offers hepatoprotection against DMN-induced hepatotoxicity in rats by restoring the altered levels of the serum and liver marker enzymes, by preventing the elevation in the status of lipid peroxidation, and bringing back the levels of both the enzymatic and non-enzymatic antioxidants towards normalcy and this is attributed to its free radical scavenging and antioxidant properties.

Conflict of interest
The authors report no conflicts of interest in this regard.

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References


